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(54) Title: MUTANT α-AMYLASE COMPRISING MODIFICATION AT RESIDUES CORRESPONDING TO A210, H405 AND/OR T412 IN *BACILLUS LICHENIFORMIS*

(57) Abstract

Alpha-amylase enzymes are disclosed in which one or more of residues corresponding to A210, H405 and T412 in *Bacillus licheniformis* are mutated. The disclosed alpha-amylase enzymes show altered or improved stability and/or activity profiles.

MUTANT α-AMYLASE COMPRISING MODIFICATION AT RESIDUES CORRESPONDING TO A210, H405 AND/OR T412 IN *BACILLUS LICHENIFORMIS*

FIELD OF THE INVENTION

The present invention is directed to α-amylases having introduced therein mutations providing additional stability under certain conditions. It is specifically contemplated that the mutant will have altered performance characteristics such as altered stability and/or altered activity profiles.

BACKGROUND OF THE INVENTION

α-Amylases (α-1,4-glucan-4-glucanohydrolase, EC 3.2.1.1) hydrolyze internal α-1,4-glucosidic linkages in starch, largely at random, to produce smaller molecular weight malto-dextrins. α-Amylases are of considerable commercial value, being used in the initial stages (liquefaction) of starch processing; in alcohol production; as cleaning agents in detergent matrices; and in the textile industry for starch desizing. α-Amylases are produced by a wide variety of microorganisms including *Bacillus* and *Aspergillus*, with most commercial amylases being produced from bacterial sources such as *Bacillus licheniformis, Bacillus amyloliquefaciens, Bacillus subtilis*, or *Bacillus stearothermophilus*. In recent years, the preferred enzymes in commercial use have been those from *Bacillus licheniformis* because of their heat stability and performance under commercial operating conditions.

In general, starch to fructose processing consists of four steps: liquefaction of granular starch, saccharification of the liquefied starch into dextrose, purification, and isomerization to fructose. The object of a starch liquefaction process is to convert a concentrated suspension of starch polymer granules into a solution of soluble shorter chain length dextrins of low viscosity. This step is essential for convenient handling with standard equipment and for efficient conversion to glucose or other sugars. To liquefy granular starch, it is necessary to gelatinize the granules by raising the temperature of the granular starch to over about 72°C. The heating process instantaneously disrupts the insoluble starch granules to produce a water soluble starch solution. The solubilized starch solution is then liquefied by α-amylase (EC 3.2.1.1.).

A common enzymatic liquefaction process involves adjusting the pH of a granular starch slurry to between 6.0 and 6.5, the pH optimum of α-amylase derived from *Bacillus licheniformis*, with the addition of calcium hydroxide, sodium hydroxide or sodium carbonate. The addition of calcium hydroxide has the advantage of also providing calcium

ions which are known to stabilize the α-amylases against inactivation. Upon addition of α-amylases , the suspension is pumped through a steam jet to instantaneously raise the temperature to between 80-115°C. The starch is immediately gelatinized and, due to the presence of α-amylases, depolymerized through random hydrolysis of a(1-4) glycosidic bonds to a fluid mass which is easily pumped.

In a second variation to the liquefaction process, α-amylase is added to the starch suspension, the suspension is held at a temperature of 80-100°C to partially hydrolyze the starch granules, and the partially hydrolyzed starch suspension is pumped through a jet at temperatures in excess of about 105°C to thoroughly gelatinize any remaining granular structure. After cooling the gelatinized starch, a second addition of α-amylase can be made to further hydrolyze the starch.

A third variation of this process is called the dry milling process. In dry milling, whole grain is ground and combined with water. The germ is optionally removed by flotation separation or equivalent techniques. The resulting mixture, which contains starch, fiber, protein and other components of the grain, is liquefied using α-amylase. The general practice in the art is to undertake enzymatic liquefaction at a lower temperature when using the dry milling process. Generally, low temperature liquefaction is believed to be less efficient than high temperature liquefaction in converting starch to soluble dextrins.

Typically, after gelatinization the starch solution is held at an elevated temperature in the presence of α-amylase until a DE of 10-20 is achieved, usually a period of 1-3 hours. Dextrose equivalent (DE) is the industry standard for measuring the concentration of total reducing sugars, calculated as D-glucose on a dry weight basis. Unhydrolyzed granular starch has a DE of virtually zero, whereas the DE of D-glucose is defined as 100.

The maximum temperature at which the starch solution containing α-amylase can be held depends upon the microbial source from which the enzyme was obtained and the molecular structure of the α-amylase molecule. α-Amylases produced by wild type strains of *Bacillus subtilis* or *Bacillus amyloliquefaciens* are typically used at temperatures no greater than about 90°C due to excessively rapid thermal inactivation above that temperature, whereas α-amylases produced by wild type strains of *Bacillus licheniformis* can be used at temperatures up to about 110°C. The presence of starch and calcium ion are known to stabilize α-amylases against inactivation. Nonetheless, α-amylases are used at pH values above 6 to protect against rapid inactivation. At low temperatures, α-amylase from *Bacillus licheniformis* is known to display hydrolyzing activity on starch substrate at pH values lower than 5. However, when the enzyme is used for starch hydrolysis at common jet temperatures, e.g., between 102°C and 109°C, the pH must be maintained above at least pH 5.7 to avoid excessively rapid inactivation. The pH requirement

unfortunately provides a narrow window of processing opportunity because pH values above 6.0 result in undesirable by-products, e.g., maltulose. Therefore, in reality, liquefaction pH is generally maintained between 5.9 and 6.0 to attain a satisfactory yield of hydrolyzed starch.

Another problem relating to pH of liquefaction is the need to raise the pH of the starch suspension from about 4, the pH of a corn starch suspension as it comes from the wet milling stage, to 5.9-6.0. This pH adjustment requires the costly addition of acid neutralizing chemicals and also requires additional ion-exchange refining of the final starch conversion product to remove the chemical. Moreover, the next process step after liquefaction, typically saccharification of the liquefied starch into glucose with glucoamylase, requires a pH of 4-4.5; therefore, the pH must be adjusted down from 5.9-6.0 to 4-4.5; requiring additional chemical addition and refining steps.

Subsequent to liquefaction, the processed starch is saccharified to glucose with glucoamylase. A problem with present processes occurs when residual starch is present in the saccharification mixture due to an incomplete liquefaction of the starch, e.g., inefficient amylose hydrolysis by amylase. Residual starch is highly resistant to glucoamylase hydrolysis. It represents a yield loss and interferes with downstream filtration of the syrups.

Additionally, many α-amylases are known to require the addition of calcium ion for stability. This further increases the cost of liquefaction.

In U.S. Patent No. 5,322,778, liquefaction between pH 4.0 and 6.0 was achieved by adding an antioxidant such as bisulfite or a salt thereof, ascorbic acid or a salt thereof, erythorbic acid, or phenolic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, or a-tocopherol to the liquefaction slurry. According to this patent, sodium bisulfite must be added in a concentration of greater than 5mM.

In U.S. Patent No. 5,180,669, liquefaction between a pH of 5.0 to 6.0 was achieved by the addition of carbonate ion in excess of the amount needed to buffer the solution to the ground starch slurry. Due to an increased pH effect which occurs with addition of carbonate ion, the slurry is generally neutralized by adding a source of hydrogen ion, for example, an inorganic acid such as hydrochloric acid or sulfuric acid.

In PCT Publication No. WO 95/35382, a mutant α-amylase is described having improved oxidation stability and having changes at positions 104, 128, 187 and/or 188 in *B. licheniformis* α-amylase.

In PCT Publication No. WO 96/23873, mutant α-amylases are described which have any of a number of mutations.

In PCT Publication No. WO 94/02597, a mutant α-amylase having improved oxidative stability is described wherein one or more methionines are replaced by any amino acid except cysteine or methionine.

In PCT publication No. WO 94/18314, a mutant α-amylase having improved oxidative stability is described wherein one or more of the methionine, tryptophan, cysteine, histidine or tyrosine residues is replaced with a non-oxidizable amino acid.

In PCT Publication No. WO 91/00353, the performance characteristics and problems associated with liquefaction with wild type *Bacillus licheniformis* α-amylase are approached by genetically engineering the α-amylase to include the specific substitutions Ala-111-Thr, His-133-Tyr and/or Thr-149-Ile.

Studies using recombinant DNA techniques to explore which residues are important for the catalytic activity of amylases and/or to explore the effect of modifying certain amino acids within the active site of various amylases and glycosylases have been conducted by various researchers (Vihinen et al., J. Biochem., Vol. 107, pp. 267-272 (1990); Holm et al., Protein Engineering, Vol. 3, pp. 181-191 (1990); Takase et al., Biochemica et Biophysica Acta, Vol. 1120, pp. 281-288 (1992); Matsui et al., FEBS Letters, Vol. 310, pp. 216-218 (1992); Matsui et al., Biochemistry, Vol. 33, pp. 451-458 (1992); Sogaard et al., J. Biol. Chem., Vol. 268, pp. 22480-22484 (1993); Sogaard et al., Carbohydrate Polymers, Vol. 21, pp. 137-146 (1993); Svensson, Plant Mol. Biol., Vol. 25, pp. 141-157 (1994); Svensson et al., J. Biotech., Vol. 29, pp. 1-37 (1993)). Researchers have also studied which residues are important for thermal stability (Suzuki et al., J. Biol. Chem. Vol. 264, pp. 18933-18938 (1989); Watanabe et al., Eur. J. Biochem., Vol. 226, pp. 277-283 (1994)); and one group has used such methods to introduce mutations at various histidine residues in a *Bacillus licheniformis* amylase, the rationale being that *Bacillus licheniformis* amylase which is known to be relatively thermostable when compared to other similar *Bacillus* amylases, has an excess of histidines and, therefore, it was suggested that replacing a histidine could affect the thermostability of the enzyme. This work resulted in the identification of stabilizing mutations at the histidine residue at the +133 position and the alanine residue at position +209 (Declerck et al., J. Biol. Chem., Vol. 265, pp. 15481-15488 (1990); FR 2 665 178-A1; Joyet et al., Bio/Technology, Vol. 10, pp. 1579-1583 (1992)).

Despite the advances made in the prior art, a need exists for an α-amylase which is more effective in commercial liquefaction processes but allowing activity at lower pH than currently practical. Additionally, a need exists for improved amylases having characteristics which makes them more effective under the conditions of detergent use. Because commercially available amylases are not acceptable under many conditions due

to stability problems, for example, the high alkalinity and oxidant (bleach) levels associated with detergents, or temperatures under which they operate, there is a need for an amylase having altered, and preferably increased, performance profiles under such conditions.

SUMMARY OF THE INVENTION

It is an object of the present invention to provide an α-amylase having altered performance profiles.

It is a further object of the present invention to provide an α-amylase having improved stability at high temperature.

Accordingly, the present invention provides an α-amylase having introduced therein a mutation comprising an addition, substitution or deletion at a residue corresponding to A210, H405 and/or T412 in *Bacillus licheniformis* α-amylase. In a particularly preferred embodiment of the invention, the α-amylase is derived from a bacterial or a fungal source and comprises a substitution corresponding to *Bacillus licheniformis.* Most preferably, the α-amylase is derived from *Bacillus* and the mutations correspond to A210T, H405D and/or T412A in *Bacillus licheniformis.*

The invention further comprises nucleic acids encoding such mutant amylases, vectors comprising such nucleic acids, host cells transformed with such vectors and methods of expressing mutant α-amylases utilizing such host cells.

The invention further comprises the use of the mutant α-amylases according to the invention to liquefy starch in the starch processing pathway to glucose or other starch derivatives, as an additive in detergents such as laundry and dishwashing detergents, as a baking aid and for desizing of textiles.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the DNA sequence of the gene for α-amylase from *Bacillus licheniformis* (NCIB 8061) (SEQ ID NO:1) and deduced amino acid sequence of the translation product (SEQ ID NO:2) as described by Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986).

Figure 2 illustrates the amino acid sequence (SEQ ID NO:3) of the mature α-amylase enzyme from *Bacillus licheniformis*.

Figure 3 illustrates an alignment of the primary structures of three *Bacillus* α-amylases. The *Bacillus licheniformis* α-amylase (Am-Lich) (SEQ ID NO:4) is described by Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986); the *Bacillus amyloliquefaciens* α-amylase (Am-Amylo) (SEQ ID NO:5) is described by Takkinen et al., J. Biol. Chem., Vol.

258, pp. 1007-1013 (1983); and the *Bacillus stearothermophilus* α-amylase (Am-Stearo) (SEQ ID NO:6) is described by Ihara et al., J. Biochem., Vol. 98, pp. 95-103 (1985).

Figure 4 illustrates plasmid pHP13 wherein Cm^R refers to chloramphenicol resistance, Em^R refers to erythromycin resistance and Rep pTA1060 refers to the origin of replication from plasmid pTA1060.

Figure 5 illustrates the pBLapr plasmid wherein BL AA refers to *Bacillus licheniformis* α-amylase gene; *aprE* refers to the promoter and signal peptide encoding region of the *aprE* gene; AmpR refers to the ampicillin resistant gene from pBR322; and CAT refers to the chloramphenicol resistance gene from pC194.

Figure 6 illustrates the pHP.BL plasmid carrying the gene for *Bacillus licheniformis* α-amylase.

DETAILED DESCRIPTION

"α-Amylase" means an enzymatic activity which cleaves or hydrolyzes the α(1-4)glycosidic bond, e.g., that in starch, amylopectin or amylose polymers. α-Amylase as used herein includes naturally occurring α-amylases as well as recombinant α-amylases. Preferred α-amylases in the present invention are those derived from *Bacillus licheniformis, Bacillus amyloliquefaciens* or *Bacillus stearothermophilus*, as well as fungal α-amylases such as those derived from *Aspergillus* (i.e., *A. oryzae* and *A. niger*).

"Recombinant α-amylase" means an α-amylase in which the DNA sequence encoding the naturally occurring α-amylase is modified to produce a mutant DNA sequence which encodes the substitution, insertion or deletion of one or more amino acids in the α-amylase sequence compared to the naturally occurring α-amylase.

"Expression vector" means a DNA construct comprising a DNA sequence which is operably linked to a suitable control sequence capable of effecting the expression of said DNA in a suitable host. Such control sequences may include a promoter to effect transcription, an optional operator sequence to control such transcription, a sequence encoding suitable mRNA ribosome-binding sites, and sequences which control termination of transcription and translation. A preferred promoter is the *Bacillus subtilis aprE* promoter. The vector may be a plasmid, a phage particle, or simply a potential genomic insert. Once transformed into a suitable host, the vector may replicate and function independently of the host genome, or may, in some instances, integrate into the genome itself. In the present specification, plasmid and vector are sometimes used interchangeably as the plasmid is the most commonly used form of vector at present.

However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which are, or become, known in the art.

"Host strain" or "host cell" means a suitable host for an expression vector comprising DNA encoding the α-amylase according to the present invention. Host cells useful in the present invention are generally procaryotic or eucaryotic hosts, including any transformable microorganism in which the expression of α-amylase according to the present invention can be achieved. Specifically, host strains of the same species or genus from which the α-amylase is derived are suitable, such as a *Bacillus* strain. Preferably, an α-amylase negative *Bacillus* strain (genes deleted) and/or an α-amylase and protease deleted *Bacillus* strain (Δ*amyE*, Δ*apr*, Δ*npr*) is used. Host cells are transformed or transfected with vectors constructed using recombinant DNA techniques. Such transformed host cells are capable of either replicating vectors encoding the α-amylase and its variants (mutants) or expressing the desired α-amylase.

"Liquefaction" or "liquefy" means a process by which starch is converted to shorter chain and less viscous dextrins. Generally, this process involves gelatinization of starch simultaneously with or followed by the addition of α-amylase.

According to the present invention, a mutant α-amylase is provided that has introduced therein a substitution, addition or deletion at A210, H405 and/or T412. Deletion, addition or substitution of an amino acid as used herein refers to any modification of the amino acid sequence of the precursor α-amylase itself, but preferably refers to using genetic engineering to mutate a nucleic acid encoding the precursor α-amylase so as to encode the deleted, substituted or added residue in the expressed protein. The precursor α-amylases include naturally occurring α-amylases and recombinant α-amylases. Modification of the precursor DNA sequence which encodes the amino acid sequence of the precursor α-amylase can be by methods described herein and in commonly owned U.S. Patent Nos. 4,760,025 and 5,185,258, incorporated herein by reference.

Also provided is a nucleic acid molecule (DNA) which encodes an amino acid sequence comprising the mutant α-amylase provided by the present invention, expression systems incorporating such DNA including vectors and phages, host cells transformed with such DNA, and anti-sense strands of DNA corresponding to the DNA molecule which encodes the amino acid sequence. Similarly, the present invention includes a method for producing a mutant α-amylase by expressing the DNA incorporated in an expression system which has been transformed into a host cell. The mutant α-amylase of the invention may be used in liquefaction of starch, as an ingredient in laundry detergents,

automatic dishwashing detergents, hard surface cleaning products, in food processing including baking applications, in textile processing including as a desize agent, or in any other application in which α-amylase activity is useful.

The precursor α-amylase is produced by any source capable of producing α-amylase. Suitable sources of α-amylases are prokaryotic or eukaryotic organisms, including fungi, bacteria, plants or animals. Preferably, the precursor α-amylase is produced by a *Bacillus*; more preferably, by *Bacillus licheniformis, Bacillus amyloliquefaciens* or *Bacillus stearothermophilus;* most preferably, the precursor α-amylase is derived from *Bacillus licheniformis.*

Homologies have been found between almost all endo-amylases sequenced to date, ranging from plants, mammals, and bacteria (Nakajima et al., Appl. Microbiol. Biotechnol., Vol. 23, pp. 355-360 (1986); Rogers, Biochem. Biophys. Res. Commun., Vol. 128, pp. 470-476 (1985); Janecek, Eur. J. Biochem., Vol. 224, pp. 519-524 (1994)). There are four areas of particularly high homology in certain *Bacillus* amylases, as shown in Figure 3, wherein the underlined sections designate the areas of high homology. Sequence alignments have also been used to map the relationship between *Bacillus* endo-amylases (Feng et al., J. Molec. Evol., Vol. 35, pp. 351-360 (1987)). The relative sequence homology between *Bacillus stearothermophilus* and *Bacillus licheniformis* amylase is about 66% and that between *Bacillus licheniformis* and *Bacillus amyloliquefaciens* amylases is about 81%, as determined by Holm et al., Protein Engineering, Vol. 3, No. 3, pp. 181-191 (1990). While sequence homology is important, it is generally recognized that structural homology is also important in comparing amylases or other enzymes. For example, structural homology between fungal amylases and bacterial amylase has been suggested and, therefore, fungal amylases are encompassed within the present invention.

Among others, addition, deletion or substitution at residues corresponding to A210, H405 and/or T412 in *Bacillus licheniformis* α-amylase are identified herein. Thus, specific residues such as A210 refer to an amino acid position number (i.e., +210) which references the number assigned to the mature *Bacillus licheniformis* α-amylase sequence illustrated in Figure 1. The invention, however, is not limited to the mutation of the particular mature α-amylase of *Bacillus licheniformis* but extends to precursor α-amylases containing amino acid residues at positions which are equivalent to the particular identified residue in *Bacillus licheniformis* α-amylase. A residue of a precursor α-amylase is equivalent to a residue of *Bacillus licheniformis* α-amylase if it is either homologous (i.e., corresponds in position for either the primary or tertiary structure) or analogous to a

specific residue or portion of that residue in *Bacillus licheniformis* α-amylase (i.e., having the same or similar functional capacity to combine, react, or interact chemically or structurally).

In order to establish homology to primary structure, the amino acid sequence of a precursor α-amylase is directly compared to the *Bacillus licheniformis* α-amylase primary sequence and particularly to a set of residues known to be invariant to all α-amylases for which sequences are known (see e.g., Figure 3). It is possible also to determine equivalent residues by tertiary structure analysis of the crystal structures reported for porcine pancreatic α-amylase (Buisson et al., EMBO Journal, Vol. 6, pp. 3909-3916 (1987); Qian et al., Biochemistry, Vol. 33, pp. 6284-6294 (1994); Larson et al., J. Mol. Biol., Vol. 235, pp. 1560-1584 (1994)); Taka-amylase A from *Aspergillus oryzae* (Matsuura et al., J. Biochem. (Tokyo), Vol. 95, pp. 697-702 (1984)); and an acid α-amylase from *A. niger* (Boel et al.. Biochemistry, Vol. 29, pp. 6244-6249 (1990)), with the former two structures being similar, and for barley α-amylase (Vallee et al., J. Mol. Biol., Vol. 236, pp. 368-371(1994); Kadziola, J. Mol. Biol., Vol. 239, pp. 104-121 (1994)). Several preliminary studies have been published related to the secondary structure of α-amylase, i.e., (Suzuki et al., J. Biochem., Vol. 108, pp. 379-381 (1990); Lee et al., Arch. Biochem. Biophys, Vol. 291, pp. 255-257 (1991); Chang et al., J. Mol. Biol., Vol. 229, pp. 235-238 (1993); Mizuno et al., J. Mol. Biol., Vol. 234, pp. 1282-1283 (1993)), and at least one structure has been published for crystalline *Bacillus licheniformis* α-amylase (Machius et al., J. Mol. Biol. Vol. 246, pp. 545-549 (1995)). However, several researchers have predicted common super-secondary structures between glucanases (MacGregor et al., Biochem. J., Vol. 259, pp. 145-152 (1989)) and within α-amylases and other starch-metabolising enzymes (Jaspersen, J. Prot. Chem. Vol. 12, pp. 791-805 (1993); MacGregor, Starke, Vol. 45, pp. 232-237 (1993)); and sequence similarities between enzymes with similar super-secondary structures to α-amylases (Janecek, FEBS Letters, Vol. 316, pp. 23-26 (1993); Janecek et al., J. Prot. Chem., Vol. 12, pp. 509-514 (1993)). A structure for the *Bacillus stearothermophilus* enzyme has been modeled on that of Taka-amylase A (Holm et al., Protein Engineering, Vol. 3, pp. 181-191 (1990)). The four highly conserved regions shown in Figure 3 contain many residues thought to be part of the active-site (Matsuura et al., J. Biochem. (Tokyo), Vol. 95, pp. 697-702 (1984); Buisson et al., EMBO Journal, Vol. 6, pp. 3909-3916 (1987); Vihinen et al., J. Biochem., Vol. 107, pp. 267-272 (1990)) including His +105; Arg +229; Asp +231; His +235; Glu +261 and Asp +328 under the *Bacillus licheniformis* numbering system.

α-Amylases according to the present invention which exhibit altered performance characteristics providing desirable and unexpected results are useful in the various applications for which α-amylases are commonly used. For example, α-amylases according to the present invention which exhibit altered performance characteristics at low pH, including improved thermostability, improved pH stability and/or improved oxidative stability, are useful in low pH liquefaction of starch. Enhanced thermostability will be useful in extending the shelf life of products which incorporate them. Enhanced oxidative stability or improved performance is particularly desirable in cleaning products, and for extending the shelf life of α-amylase in the presence of bleach, perborate, percarbonate or peracids used in such cleaning products. To the contrary, reduced thermal stability or oxidative stability may be useful in industrial processes which require the rapid and efficient quenching of amylolytic activity.

α-Amylases of the present invention which exhibit improved low pH stability will be especially useful in starch processing and particularly in starch liquefaction. Conditions present during commercially desirable liquefaction processes characteristically include low pH, high temperature and potential oxidation conditions requiring α-amylases exhibiting improved low pH performance, improved thermal stability and improved oxidative stability. Accordingly, α-amylases according to the present invention which are particularly useful in liquefaction exhibit improved performance at a pH of less than about 6, preferably less than about 5.5, and most preferably less than about 5.0. Additionally, α-amylases according to the present invention which exhibit increased thermal stability at temperatures of between about 80-120°C, and preferably between about 100-110°C, and increased stability in the presence of oxidants will be particularly useful.

Additional components known by those skilled in the art to be useful in liquefaction, including, for example, antioxidants, calcium, ions, salts or other enzymes such as endoglycosidases, cellulases, proteases, lipases or other amylase enzymes may be added depending on the intended reaction conditions. For example, combinations of the α-amylase according to the present invention with α-amylases from other sources may provide unique action profiles which find particular use under specific liquefaction conditions. In particular, it is contemplated that the combination of the α-amylase according to the present invention with α-amylase derived from *Bacillus stearothermophilus* will provide enhanced liquefaction at pH values below 5.5 due to complementary action patterns.

During liquefaction, starch, specifically granular starch slurries from either a wet or dry milled process, is treated with an α-amylase of the present invention according to

known liquefaction techniques. Generally, in the first step of the starch degradation process, the starch slurry is gelatinized by heating at a relatively high temperature (between about 80°C and about 110°C). After the starch slurry is gelatinized, it is liquefied using an α-amylase.

In another embodiment of the present invention, detergent compositions in either liquid, gel or granular form, which comprise the α-amylase according to the present invention may be useful. Such detergent compositions will particularly benefit from the addition of an α-amylase according to the present invention which has increased thermal stability to improve shelf-life or increased oxidative stability such that the α-amylase has improved resistance to bleach or peracid compounds commonly present in detergents. Thus, α-amylase according to the present invention may be advantageously formulated into known powdered, liquid or gel detergents having a pH of between about 6.5 and about 12.0. Detergent compositions comprising the α-amylase according to the present invention may further include other enzymes such as endoglycosidases, cellulases, proteases, lipases or other amylase enzymes, particularly α-amylase derived from *Bacillus stearothermophilus,* as well as additional ingredients as generally known in the art.

A preferred embodiment of the present invention further comprises, in addition to the substitution, addition or deletion of residues as provided herein, any one or more of the substitutions known in the art to confer stability or increased activity. For example, the deletion or substitution of a methionine residue or a tryptophan residue, for example M15, M197 or W138 as described in WO 94/18314, the disclosure of which is incorporated by reference; substitution at H133Y as described in PCT Publication No. WO 91/00353; or substitution at A209 as described in DeClerck, et al., J. Biol. Chem., Vol. 265, pp. 15481-15488 (1990); or any of the substitutions described in PCT Publication Nos. WO 95/10603, WO 96/23873 and WO 96/23874. In particularly preferred embodiments, the α-amylase according to the present invention may further comprise a deletion or substitution at one or more residues corresponding to M15, A33, A52, S85, N96, V129, H133, S148, S187, N188, A209, A269 and/or A379 in *Bacillus licheniformis*.

Embodiments of the present invention which comprise a combination of the α-amylase according to the present invention with protease enzymes preferably include oxidatively stable proteases such as those described in U.S. Re. 34,606, incorporated herein by reference, as well as commercially available enzymes such as DURAZYM (Novo Nordisk) and PURAFECT® OxP (Genencor International, Inc.). Methods for making such protease mutants (oxidatively stable proteases), and particularly such mutants having a substitution for the methionine at a position equivalent to M222 in *Bacillus amyloliquefaciens*, are described in U.S. Re. 34,606.

An additional embodiment of the present invention comprises DNA encoding an α-amylase according to the present invention and expression vectors comprising such DNA. The DNA sequences may be expressed by operably linking them to an expression control sequence in an appropriate expression vector and employing that expression vector to transform an appropriate host according to well known techniques. A wide variety of host/expression vector combinations may be employed in expressing the DNA sequences of this invention. Useful expression vectors, for example, include segments of chromosomal, non-chromosomal and synthetic DNA sequences, such as the various known plasmids and phages useful for this purpose. In addition, any of a wide variety of expression control sequences are generally used in these vectors. For example, Applicants have discovered that a preferred expression control sequence for *Bacillus* transformants is the *aprE* signal peptide derived from *Bacillus subtilis.*

A wide variety of host cells are also useful in expressing the DNA sequences of this invention. These hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli, Pseudomonas, Bacillus, Streptomyces,* various fungi, yeast and animal cells. Preferably, the host expresses the α-amylase of the present invention extracellularly to facilitate purification and downstream processing. Expression and purification of the mutant α-amylase of the invention may be effected through art-recognized means for carrying out such processes.

The improved α-amylases according to the present invention are contemplated to provide important advantages when compared to wild type *Bacillus* α-amylases. For example, one advantage is the increased activity found at low pH and high temperatures typical of common starch liquefaction methods. Other advantages may include increased high pH and oxidative stability which facilitates their use in detergents; more complete hydrolysis of starch molecules is achieved which reduces residual starch in the processing stream; improved stability in the absence of calcium ion; and that the addition of equal protein doses of α-amylase according to the invention may provide superior performance when compared to wild type *Bacillus licheniformis* α-amylase due to improvements in both specific activity and stability under stressed conditions.

The following is presented by way of example and is not to be construed as a limitation to the scope of the claims. Abbreviations used herein, particularly three letter or one letter notations for amino acids are described in Dale, J.W., Molecular Genetics of Bacteria, John Wiley & Sons, (1989) Appendix B.

EXAMPLES

EXAMPLE 1
Construction Of Plasmid pHP.BL

The α-amylase gene shown in Figure 1 was cloned from *Bacillus licheniformis* NCIB8061 (Gray et al., J. Bacteriology, Vol. 166, pp. 635-643 (1986)). The 1.72kb PstI-SstI fragment, encoding the last three residues of the signal sequence, the entire mature protein and the terminator region, was subcloned into M13mp18. A synthetic terminator was added between the BclI and SstI sites using a synthetic oligonucleotide cassette of the form:

5'-GATCAAAACATAAAAAACCGGCCTTGGCCCCGCCGGTTTTTTATTATTTTTGAGCT-3' (SEQ ID NO:7)
3'-TTTTGTATTTTTTGGCCGGAACCGGGGCGGCCAAAAAATAATAAAAAC-5' (SEQ ID NO:8)

designed to contain the *Bacillus amyloliquefaciens* subtilisin transcriptional terminator (Wells et al., Nucleic Acid Research, Vol. 11, pp. 7911-7925 (1983)).

The pBLapr plasmid was constructed carrying the gene for the *Bacillus licheniformis* α-amylase. As illustrated in Figure 5, pBLapr comprises a 6.1kb plasmid including the ampicillin resistance gene from pBR322 and the chloramphenicol resistance gene from pC194, the aprE promoter and the gene encoding for the *Bacillus licheniformis* α-amylase ("BL AA"). The aprE promoter was constructed from a 660bp HindIII-PstI fragment encoding for the promoter and signal sequence of the *Bacillus subtilis* alkaline protease. The PstI site was removed, and an SfiI site added close to the *aprE*/BL AA junction. The BL AA gene comprises the 1720 bp PstI-SstI fragment described above. In the work described herein, pBLapr was constructed with an SfiI site adjacent to the 5' end of the start of the coding sequence for the mature amylase gene. Specifically, the 5' end of the pBLapr construction was subcloned on an EcoRI-SstII fragment from pBLapr into M13BM20 (Boehringer Mannheim) to obtain a coding-strand template for the mutagenic oligonucleotide below:

5'- CCC ATT AAG ATT GGC CGC CTG GGC CGA CAT GTT GCT GG - 3' (SEQ ID NO:9)

This primer introduced an SfiI site (indicated by underlining) which allowed correct forms to be screened for by the presence of this unique restriction site. Subcloning the EcoRI-SstII fragment back into the pBLapr vector gave a version of the plasmid containing an SfiI site.

Plasmid pHP13 (Haima et al., Mol. Gen. Genet., Vol. 209, pp. 335-342 (1987)) (Figure 4) was digested with restriction enzymes EcoRI and HindIII and the resulting vector purified on a polyacrymide gel and then eluted. Plasmid pBLapr was digested with HindIII,

Asp718 and in a separate incubation with Asp718, EcoRI and gel purified. Two bands, HindIII-Asp718 (1203 bp) and Asp718-EcoRI (1253 bp)were gel purified, eluted from the gel and ligated into the vector by a 3-way ligation, to give plasmid pHP.BL, the plasmid used in expression of the α-amylase (Figure 6).

EXAMPLE 2

Construction Of Plasmid Encoding α-Amylase Comprising A210T/H405A/T412D

A pBLapr plasmid having threonine substituted for methionine at amino acid 15 was constructed according to U.S. Patent Application Serial No. 08/194,664 (PCT Publication No. WO 94/18314). To introduce the mutations, the following mutagenic primers encoding for substitutions of A210T/M405D/T412A are used together with non-mutagenic primers to introduce the desired mutations into linear multiple tandem repeats of the plasmid by the method of multimerization as described below.

H405D (L)
(411) CCA GCC GAC AAT GTC ATG GTC GTC GAA ATA ATC (401) (SEQ ID NO:10)
A210T (R)
(206) CCT GAT GTC GCA ACA GAA ATT AAG AGA TGG (215) (SEQ ID NO:11)
T412A (L)
(416) GTC GCC TTC CCT TGC CCA GCC GAC AAT GTC (407) (SEQ ID NO:12)

A fragment starting at the appropriate mutagenic primer for the desired mutation (shown above) and ending at the end of the non-mutagenic primer is generated by PCR. This fragment is gel purified and used to generate long, linear tandem repeats of the plasmid encoding the desired mutations as follows:

The vector (pBLapr) is linearized by restriction digest (Sal I) and purified using Qiagen kits. The multimerization reactions typically contain 5.4 mM Tris buffer pH 8.0, 1x XL buffer (Perkin Elmer, Branchburg, NJ), 0.2 mM dNTPs, 1.1 mM $Mg(OAc)_2$, 3 ng/μl incoming fragment, 0.15 ng/μl linearized vector, 4 U *rTth* DNA polymerase, XL (Perkin Elmer) in 100 μl reaction mixture. PCR reactions are typically performed in a thermocycler under the following conditions: 20 cycles (15s 94°C, 5 min 68°C) and 15 cycles (15s 94°C, 10 min 68°C).

The resulting multimers are transformed directly into *B. subtilis* competent cells using standard techniques. Plasmid DNA is isolated from the transformants using standard techniques.

Mutations were confirmed by dideoxy sequencing (Sanger et al., Proc. Natl. Acad.

Sci. U.S.A., Vol. 74, pp. 5463-5467 (1977)).

EXAMPLE 3

Transformation Of Plasmids Into *Bacillus subtilis*, Expression And Purification of Mutant α-Amylase

α-Amylase may be expressed in *Bacillus subtilis* after transformation with the plasmids described above. pHP13 is a plasmid able to replicate in *E. coli* and in *Bacillus subtilis*. Plasmids containing different variants were constructed using *E. coli* strain MM294, the plasmids isolated and then transformed into *Bacillus subtilis* as described in Anagnostopoulos et al., J. Bacter., Vol. 81, pp. 741-746 (1961). The *Bacillus* strain had been deleted for two proteases (Δapr, Δnpr) (see e.g., Ferrari et al., U.S. Patent No. 5,264,366) and for amylase (Δ*amyE*) (see e.g., Stahl et al., J. Bacter., Vol. 158, pp. 411-418 (1984)). After transformation, the sacU(Hy) mutation (Henner et al., J. Bacter., Vol., 170, pp. 296-300 (1988)) was introduced by PBS-1 mediated transduction (Hoch, J. Bact., Vol. 154, pp. 1513-1515 (1983)).

Secreted amylase was recovered from *Bacillus subtilis* cultures as follows: Sodium chloride was added to the culture supernatant to 20mM and the pH was adjusted to approximately 7.0 with 1M tris buffer, pH 7.2. The supernatant was then heated to 70°C for 15 minutes, and the precipitate removed by centrifugation. Ammonium sulphate was added the supernatant to 1.3M followed by 20ml phenyl sepharose fast flow 6 (high substitution) resin (Pharmacia). Following agitation, resin was separated by filtration, and washed in 1M ammonium sulphate, 20mM ammonium acetate pH 7.0, 5mM calcium chloride. The bound amylase was eluted into 20mM ammonium acetate pH 7.0, 5mM calcium chloride, and precipated by addition of ammonium sulphate to 70% saturation. The precipated material was pelleted by centrifugation, redissolved in a minimum volume of 20mM ammonium acetate pH 7.0, 5mM calcium chloride and dialysed against the same buffer.

Concentration was determined using the soluble substrate assay, assuming wild-type specific activity.

EXAMPLE 4

Assay For Determining α-Amylase Activity

Soluble Substrate Assay: A rate assay was developed based on an end-point assay kit supplied by Megazyme (Aust.) Pty. Ltd. A vial of substrate (*p*-nitrophenyl maltoheptaoside, BPNPG7) was dissolved in 10ml of sterile water followed by a 1:4 dilution in assay buffer (50mM maleate buffer, pH 6.7, 5mM calcium chloride, 0.002%

Tween20). Assays were performed by adding 10μl of amylase to 790μl of the substrate in a cuvette at 25°C. Rates of hydrolysis were measured as the rate of change of absorbance at 410nm, after a delay of 75 seconds. The assay was linear up to rates of 0.2 absorption units/min.

α-Amylase protein concentration was measured using the standard Bio-Rad Assay (Bio-Rad Laboratories) based on the method of Bradford, Anal. Biochem., Vol. 72, p. 248 (1976) using bovine serum albumin standards.

EXAMPLE 5

Preparation and Testing of Additional Mutant Alpha-Amylases for Thermal Stability

Mutant *B. licheniformis* alpha-amylase was prepared having substitutions at A210T/H405D/T412A. Thermal inactivation rate for the mutant was measured according to the following procedure. Amylase stock solutions were dialysed extensively into 20 mM ammonium acetate, 4 mM $CaCl_2$ pH 6.5. Each sample was stored at 4°C. For measurement of stability, this stock was diluted >50fold into 50mM ammonium acetate, 5mM $CaCl_2$, 0.02% Tween 20 pH 4.8 to a final concentration of between 30 and 50 μg/ml. Six 100μl aliquots were put into eppendorf tubes and placed into a water bath or hot block at 83°C. The eppendorf tubes were removed at regular, measured intervals of between 30 seconds and 5 minutes and placed on ice to stop the inactivation. The residual activity was assayed using a soluble substrate as described in Example 4. The natural log of the activity was plotted against time of incubation, and the rate constant for inactivation obtained from the slope of the straight line. Results are provided in Table 1.

TABLE 1

	Relative Half-Life	
Amylase	Exp. #1	Exp. #2
wild type	1.00	1.00
wild type	1.01	XX
A210T/H405D/T412A	1.06	1.05

As shown in Table 1, mutant enzymes having introduced therein the mutations according to the invention have significantly improved stability under the conditions of the assay.

CLAIMS

1. A mutant α-amylase which is derived from a precursor α-amylase by the deletion, substitution or addition to said precursor α-amylase of a residue corresponding to A210, H405 and/or T412 in *Bacillus licheniformis* α-amylase.

2. The mutant α-amylase according to claim 1, wherein said mutation comprises deletion, substitution or addition at two or more of A210, H405 and/or T412 in *Bacillus licheniformis* α-amylase.

3. The α-amylase according to claim 2, wherein said α-amylase comprises a substitution corresponding to A210T/H405D/T412A in *Bacillus licheniformis* α-amylase.

4. The α-amylase according to claim 1, wherein said α-amylase is derived from a bacterial or fungal source.

5. The α-amylase according to claim 1, wherein said α-amylase is derived from *Bacillus*.

6. The α-amylase according to claim 5, wherein said α-amylase is derived from *Bacillus licheniformis, Bacillus stearothermophilus* or *Bacillus amyloliquefaciens.*

7. The α-amylase according to claim 1 wherein said α-amylase further comprises the deletion or substitution of a residue corresponding to M15, A33, A52, S85, N96, V129, H133, S148N, S187, N188, A209, A269 and/or A379 in *Bacillus licheniformis* α-amylase.

8. The α-amylase according to claim 1, wherein substitution further comprises substituting or deleting a residue corresponding to M15T, W138Y and/or M197T in *Bacillus licheniformis*.

9. A DNA encoding the α-amylase according to claim 1.

10. An expression vector comprising the DNA of claim 9.

11. A host cell transformed with the expression vector of claim 10.

12. An α-amylase according to claims 1, 3 or 7 having enhanced low pH performance and/or increased thermostability.

13. A detergent composition comprising the α-amylase according to claim 1.

14. The detergent composition according to claim 13, wherein said detergent is useful for cleaning soiled laundry and/or soiled dishes.

15. A method of liquefying starch comprising contacting a slurry of starch with the α-amylase according to claim 1.

1/9

10 30 50
AGCTTGAAGAAGTGAAGAAGCAGAGAGGCTATTGAATAAATGAGTAGAAAGCGCCATATC

70 90 110
GGCGCTTTTCTTTTGGAAGAAAATATAGGGAAAATGGTACTTGTTAAAAATTCGGAATAT

130 150 170
TTATACAACATCATATGTTTCACATTGAAAGGGGAGGAGAATCATGAAACAACAAAAACG
M K Q Q K R

190 210 230
GCTTTACGCCCGATTGCTGACGCTGTTATTTGCGCTCATCTTCTTGCTGCCTCATTCTGC
L Y A R L L T L L F A L I F L L P H S A

250 270 290
AGCAGCGGCGGCAAATCTTAATGGGACGCTGATGCAGTATTTTGAATGGTACATGCCCAA
A A A A N L N G T L M Q Y F E W Y M P N

310 330 350
TGACGGCCAACATTGGAAGCGTTTGCAAAACGACTCGGCATATTTGGCTGAACACGGTAT
D G Q H W K R L Q N D S A Y L A E H G I

370 390 410
TACTGCCGTCTGGATTCCCCCGGCATATAAGGGAACGAGCCAAGCGGATGTGGGCTACGG
T A V W I P P A Y K G T S Q A D V G Y G

430 450 470
TGCTTACGACCTTTATGATTTAGGGGAGTTTCATCAAAAAGGGACGGTTCGGACAAAGTA
A Y D L Y D L G E F H Q K G T V R T K Y

490 510 530
CGGCACAAAAGGAGAGCTGCAATCTGCGATCAAAAGTCTTCATTCCCGCGACATTAACGT
G T K G E L Q S A I K S L H S R D I N V

550 570 590
TTACGGGGATGTGGTCATCAACCACAAAGGCGGCGCTGATGCGACCGAAGATGTAACCGC
Y G D V V I N H K G G A D A T E D V T A

610 630 650
GGTTGAAGTCGATCCCGCTGACCGCAACCGCGTAATTTCAGGAGAACACCTAATTAAAGC
V E V D P A D R N R V I S G E H L I K A

670 690 710
CTGGACACATTTTCATTTTCCGGGGCGCGGCAGCACATACAGCGATTTTAAATGGCATTG
W T H F H F P G R G S T Y S D F K W H W

730 750 770
GTACCATTTTGACGGAACCGATTGGGACGAGTCCCGAAAGCTGAACCGCATCTATAAGTT
Y H F D G T D W D E S R K L N R I Y K F

790 810 830
TCAAGGAAAGGCTTGGGATTGGGAAGTTTCCAATGAAAACGGCAACTATGATTATTTGAT
Q G K A W D W E V S N E N G N Y D Y L M

FIG._1A

```
      850                           870                           890
GTATGCCGACATCGATTATGACCATCCTGATGTCGCAGCAGAAATTAAGAGATGGGGCAC
 Y  A  D  I  D  Y  D  H  P  D  V  A  A  E  I  K  R  W  G  T

      910                           930                           950
TTGGTATGCCAATGAACTGCAATTGGACGGTTTCCGTCTTGATGCTGTCAAACACATTAA
 W  Y  A  N  E  L  Q  L  D  G  F  R  L  D  A  V  K  H  I  K

      970                           990                          1010
ATTTTCTTTTTTGCGGGATTGGGTTAATCATGTCAGGGAAAAAACGGGGAAGGAAATGTT
 F  S  F  L  R  D  W  V  N  H  V  R  E  K  T  G  K  E  M  F

     1030                          1050                          1070
TACGGTAGCTGAATATTGGCAGAATGACTTGGGCGCGCTGGAAAACTATTTGAACAAAAC
 T  V  A  E  Y  W  Q  N  D  L  G  A  L  E  N  Y  L  N  K  T

     1090                          1110                          1130
AAATTTTAATCATTCAGTGTTTGACGTGCCGCTTCATTATCAGTTCCATGCTGCATCGAC
 N  F  N  H  S  V  F  D  V  P  L  H  Y  Q  F  H  A  A  S  T

     1150                          1170                          1190
ACAGGGAGGCGGCTATGATATGAGGAAATTGCTGAACGGTACGGTCGTTTCCAAGCATCC
 Q  G  G  G  Y  D  M  R  K  L  L  N  G  T  V  V  S  K  H  P

     1210                          1230                          1250
GTTGAAATCGGTTACATTTGTCGATAACCATGATACACAGCCGGGGCAATCGCTTGAGTC
 L  K  S  V  T  F  V  D  N  H  D  T  Q  P  G  Q  S  L  E  S

     1270                          1290                          1310
GACTGTCCAAACATGGTTTAAGCCGCTTGCTTACGCTTTTATTCTCACAAGGGAATCTGG
 T  V  Q  T  W  F  K  P  L  A  Y  A  F  I  L  T  R  E  S  G

     1330                          1350                          1370
ATACCCTCAGGTTTTCTACGGGGATATGTACGGGACGAAAGGAGACTCCCAGCGCGAAAT
 Y  P  Q  V  F  Y  G  D  M  Y  G  T  K  G  D  S  Q  R  E  I

     1390                          1410                          1430
TCCTGCCTTGAAACACAAAATTGAACCGATCTTAAAAGCGAGAAAACAGTATGCGTACGG
 P  A  L  K  H  K  I  E  P  I  L  K  A  R  K  Q  Y  A  Y  G

     1450                          1470                          1490
AGCACAGCATGATTATTTCGACCACCATGACATTGTCGGCTGGACAAGGGAAGGCGACAG
 A  Q  H  D  Y  F  D  H  H  D  I  V  G  W  T  R  E  G  D  S

     1510                          1530                          1550
CTCGGTTGCAAATTCAGGTTTGGCGGCATTAATAACAGACGGACCCGGTGGGGCAAAGCG
 S  V  A  N  S  G  L  A  A  L  I  T  D  G  P  G  G  A  K  R

     1570                          1590                          1610
AATGTATGTCGGCCGGCAAAACGCCGGTGAGACATGGCATGACATTACCGGAAACCGTTC
 M  Y  V  G  R  Q  N  A  G  E  T  W  H  D  I  T  G  N  R  S

     1630                          1650                          1670
GGAGCCGGTTGTCATCAATTCGGAAGGCTGGGGAGAGTTTCACGTAAACGGCGGGTCGGT
 E  P  V  V  I  N  S  E  G  W  G  E  F  H  V  N  G  G  S  V
```

FIG._1B

```
        1690                         1710                         1730
TTCAATTTATGTTCAAAGATAGAAGAGCAGAGAGGACGGATTTCCTGAAGGAAATCCGTT
 S   I   Y   V   Q   R   *

        1750                         1770                         1790
TTTTTATTTTGCCCGTCTTATAAATTTCTTTGATTACATTTTATAATTAATTTTAACAAA

        1810                         1830                         1850
GTGTCATCAGCCCTCAGGAAGGACTTGCTGACAGTTTGAATCGCATAGGTAAGGCGGGGA

        1870                         1890                         1910
TGAAATGGCAACGTTATCTGATGTAGCAAAGAAAGCAAATGTGTCGAAAATGACGGTATC

        1930                         1950
GCGGGTGATCAATCATCCTGAGACTGTGACGGATGAATTGAAAAAGCT
```

FIG._1C

10 30 50
ANLNGTL**M**QYFEWY**M**PNDGQHWKRLQNDSAYLAEHGITAVWIPPAYKGTSQADVGYGAYD

70 90 110
LYDLGEFHQKGTVRTKYGTKGELQSAIKSLHSRDINVYGDVVINHKGGADATEDVTAVEV

130 150 170
DPADRNRVISGEHLIKAWTHFHFPGRGSTYSDFKWHWYHFDGTDWDESRKLNRIYKFQGK

190 210 230
AWDWEVSNENGNYDYL**M**YADIDYDHPDVAAEIKRWGTWYANELQLDGFRLDAVKHIKFSF

250 270 290
LRDWVNHVREKTGKE**M**FTVAEYWQNDLGALENYLNKTNFNHSVFDVPLHYQFHAASTQGG

310 330 350
GYD**M**RKLLNGTVVSKHPLKSVTFVDNHDTQPGQSLESTVQTWFKPLAYAFILTRESGYPQ

370 390 410
VFYGD**M**YGTKGDSQREIPALKHKIEPILKARKQYAYGAQHDYFDHHDIVGWTREGDSSVA

430 450 470
NSGLAALITDGPGGAKR**M**YVGRQNAGETWHDITGNRSEPVVINSEGWGEFHVNGGSVSIY

VQR

FIG._2

Am-Lich = *B.Licheniformis* Am-Amylo = *B.amyloliquefaciens* Am-Stearo = *B.stearothermophilus*

```
                                                              1           19
           1                                                              60
Am-Lich    ..........MKQQ KRLYARLLTL LFALIFLLPH .........SAAA AANLNGTLMQ YFEWYMPNDG
Am-Amylo   MRGRGNMIQK RKRTVSFRLV LMCTLLFVSL .........PITK TSAVNGTLMQ YFEWYTPNDG
Am-Stearo  ..........VLTF HRIIRKGWMF LLAFLLTASL FCPTGRHAKA AAPFNGTMMQ YFEWYLPDDG

                                                                          79
           61                                                             120
Am-Lich    QHWKRLQNDS AYLAEHGITA VWIPPAYKGT SQADVGYGAY DLYDLGEFHQ KGTVRTKYGT
Am-Amylo   QHWKRLQNDA EHLSDIGITA VWIPPAYKGL SQSDNGYGPY DLYDLGEFQQ KGTVRTKYGT
Am-Stearo  TLWTKVANEA NNLSSLGITA LSLPPAYKGT SRSDVGYGVY DLYDLGEFNQ KGTVRTKYGT

                                                                          139
           121                                                            180
Am-Lich    KGELQSAIKS LHSRDINVYG DVVINHKGGA DATEDVTAVE VDPADRNRVI SGEHLIKAWT
Am-Amylo   KSELQDAIGS LHSRNVQVYG DVVLNHKAGA DATEDVTAVE VNPANRNQET SEEYQIKAWT
Am-Stearo  KAQYLQAIQA AHAAGMQVYA DVVFDHKGGA DGTEWVDAVE VNPSDRNQEI SGTYQIQAWT

                                                                          197
           181                                                            240
Am-Lich    HFHFPGRGST YSDFKWHWYH FDGTDWDESR KLNRIYKF.... QGKAWDWEVS NENGNYDYLM
Am-Amylo   DFRFPGRGNT YSDFKWHWYH FDGADWDESR KISRIFKFRG EGKAWDWEVS SENGNYDYLM
Am-Stearo  KFDFPGRGNT YSSFKWRWYH FDGVDWDESR KLSRIYKFRG IGKAWDWEVD TENGNYDYLM

                                                                          257
           241                                                            300
Am-Lich    YADIDYDHPD VAAEIKRWGT WYANELQLDG FRLDAVKHIK FSFLRDWVNH VREKTGKEMF
Am-Amylo   YADVDYDHPD VVAETKKWGI WYANELSLDG FRIDAAKHIK FSFLRDWVQA VRQATGKEMF
Am-Stearo  YADLDMDHPE VVTELKNWGK WYVNTTNIDG FRLDGLKHIK FSFFPDWLSY VRSQTGKPLF

                                                                          317
           301                                                            360
Am-Lich    TVAEYWQNDL GALENYLNKT NFNHSVFDVP LHYQFHAAST QGGGYDMRKL LNGTVVSKHP
Am-Amylo   TVAEYWQNNA GKLENYLNKT SFNQSVFDVP LHFNLQAASS QGGGYDMRRL LDGTVVSRHP
Am-Stearo  TVGEYWSYDI NKLHNYITKT NGTMSLFDAP LHNKFYTASK SGGAFDMRTL MTNTLMKDQP
```

FIG._3A

```
                                                                                   377
          361                                                                      420
Am-Lich   LKSVTFVDNH DTQPGQSLES TVQTWFKPLA YAFILTRESG YPQVFYGDMY GTKGDSQREI
Am-Amylo  EKAVTFVENH DTQPGQSLES TVQTWFKPLA YAFILTRESG YPQVFYGDMY GTKGTSPKEI
Am-Stearo TLAVTFVDNH DTNPAKR..CS HGRPWFKPLA YAFILTRQEG YPCVFYGDYY GI.........PQYNI

                                                                                   437
          421                                                                      480
Am-Lich   PALKHKIEPI LKARKQYAYG AQHDYFDHHD IVGWTREGDS SVANSGLAAL ITDGPGGAKR
Am-Amylo  PSLKDNIEPI LKARKEYAYG PQHDYIDHPD VIGWTREGDS SAAKSGLAAL ITDGPGGSKR
Am-Stearo PSLKSKIDPL LIARRDYAYG TQHDYLDHSD IIGWTREGVT EKPGSGLAAL ITDGAGRSKW

                                                          483
          481                                                                      540
Am-Lich   MYVGRQNAGE TWHDITGNRS EPVVINSEGW GEFHVNGGSV SIYVQR......... .....................
Am-Amylo  MYAGLKNAGE TWYDITGNRS DTVKIGSDGW GEFHVNDGSV SIYVQK......... .....................
Am-Stearo MYVGKQHAGK VFYDLTGNRS DTVTINSDGW GEFKVNGGSV SVWVPRKTTV STIARPITTR

          541              559
Am-Lich   ........................ .................
Am-Amylo  ........................ .................
Am-Stearo PWTGEFVRWH EPRLVAWP*
```

FIG._3B

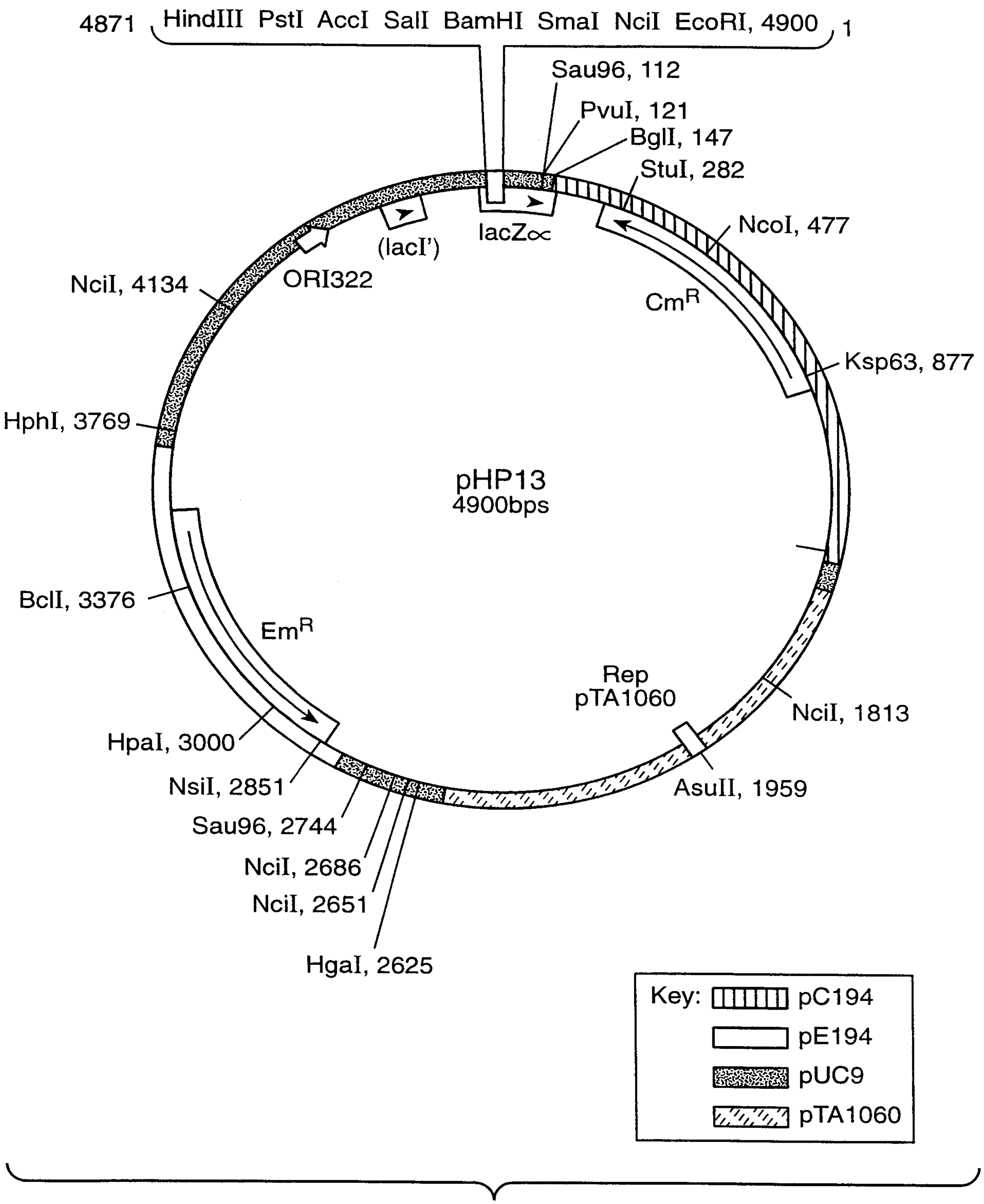

4871
HindIII PstI AccI SalI BamHI SmaI NciI EcoRI, 4900
1
Sau96, 112
PvuI, 121
BglI, 147
StuI, 282
NcoI, 477
Ksp63, 877
lacZ∝
(lacI')
ORI322
NciI, 4134
CmR
HphI, 3769
pHP13
4900bps
BclI, 3376
EmR
Rep
pTA1060
NciI, 1813
HpaI, 3000
AsuII, 1959
NsiI, 2851
Sau96, 2744
NciI, 2686
NciI, 2651
HgaI, 2625
Key:
pC194
pE194
pUC9
pTA1060

FIG._4

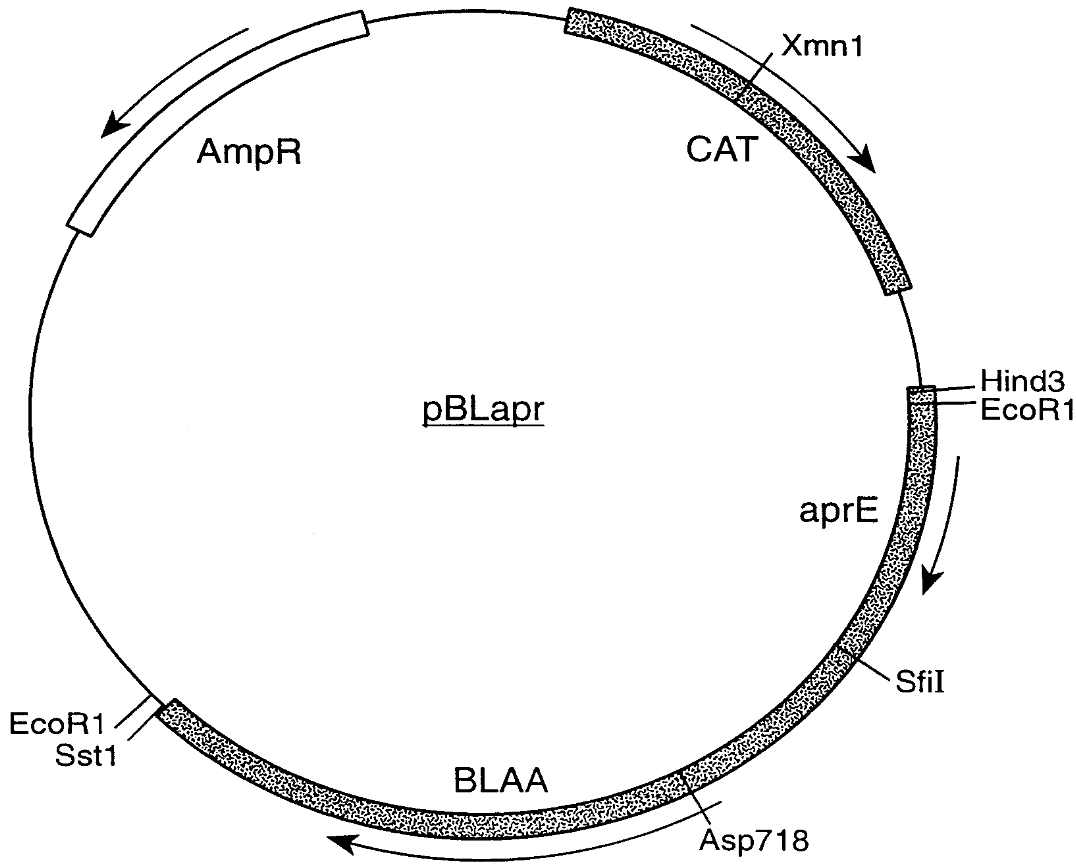
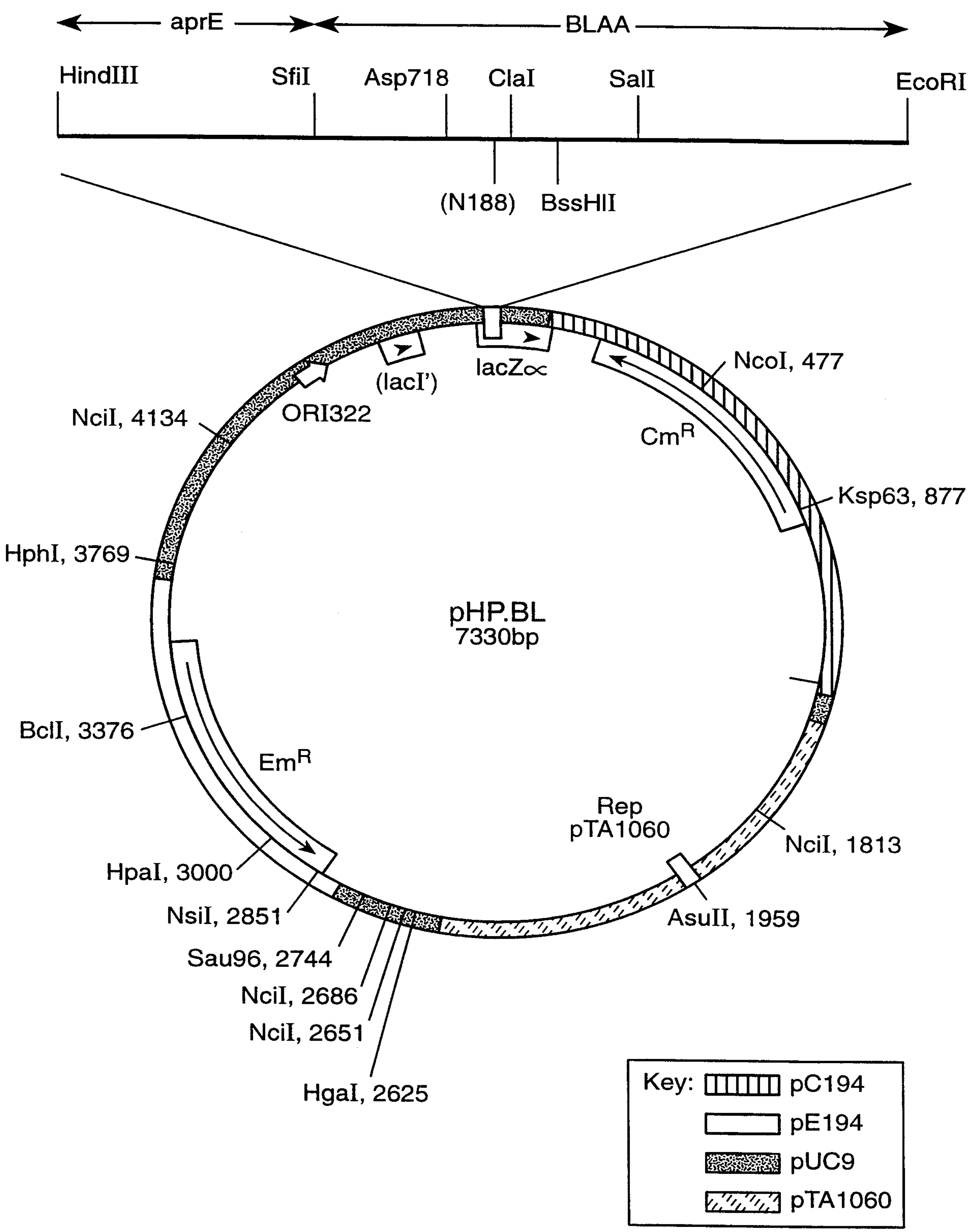

Xmn1
AmpR
CAT
pBLapr
Hind3
EcoR1
aprE
SfiI
EcoR1
Sst1
BLAA
Asp718

FIG._5



pHP.BL = pHP13 WITH 2460bp HindIII-EcoRI INSERT FROM pBLapr

FIG._6

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANTS: Anthony G. Day
Barbara A. Swanson

(ii) TITLE OF INVENTION: MUTANT ALPHA-AMYLASE COMPRISING MODIFICATION AT RESIDUES CORRESPONDING TO A210, H405 AND/OR T412 IN BACILLUS LICHENIFORMIS

(iii) NUMBER OF SEQUENCES: 12

(iv) CORRESPONDING ADDRESS:
(A) ADDRESSEE: Genencor International, Inc.
(B) STREET: 925 Page Mill Road
(C) CITY: Palo Alto
(D) STATE: CA
(E) COUNTRY: USA
(F) ZIP: 94304-1013

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER: To Be Assigned
(B) FILING DATE: To Be Assigned

(viii) ATTORNEY/AGENT INFORMATION:
(A) NAME: Stone, Christopher
(B) REGISTRATION NUMBER: 35,696
(C) REFERENCE/DOCKET NUMBER: GC387

(ix) TELECOMMUNICATION INFORMATION:
(A) TELEPHONE: (650) 846-7555
(B) TELEFAX: (650) 845-6504

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1968 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: DNA (genomic)

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

AGCTTGAAGA AGTGAAGAAG CAGAGAGGCT ATTGAATAAA TGAGTAGAAA GCGCCATATC 60

GGCGCTTTTC TTTTGGAAGA AAATATAGGG AAAATGGTAC TTGTTAAAAA TTCGGAATAT 120

TTATACAACA TCATATGTTT CACATTGAAA GGGGAGGAGA ATCATGAAAC AACAAAAACG 180

GCTTTACGCC CGATTGCTGA CGCTGTTATT TGCGCTCATC TTCTTGCTGC CTCATTCTGC 240

AGCAGCGGCG GCAAATCTTA ATGGGACGCT GATGCAGTAT TTTGAATGGT ACATGCCCAA 300

TGACGGCCAA CATTGGAAGC GTTTGCAAAA CGACTCGGCA TATTTGGCTG AACACGGTAT 360

TACTGCCGTC TGGATTCCCC CGGCATATAA GGGAACGAGC CAAGCGGATG TGGGCTACGG 420

TGCTTACGAC CTTTATGATT TAGGGGAGTT TCATCAAAAA GGGACGGTTC GGACAAAGTA 480

CGGCACAAAA GGAGAGCTGC AATCTGCGAT CAAAAGTCTT CATTCCCGCG ACATTAACGT 540

TTACGGGGAT GTGGTCATCA ACCACAAAGG CGGCGCTGAT GCGACCGAAG ATGTAACCGC 600

GGTTGAAGTC GATCCCGCTG ACCGCAACCG CGTAATTTCA GGAGAACACC TAATTAAAGC 660

CTGGACACAT TTTCATTTTC CGGGGCGCGG CAGCACATAC AGCGATTTTA AATGGCATTG 720

GTACCATTTT GACGGAACCG ATTGGGACGA GTCCCGAAAG CTGAACCGCA TCTATAAGTT 780

TCAAGGAAAG GCTTGGGATT GGGAAGTTTC CAATGAAAAC GGCAACTATG ATTATTTGAT 840

GTATGCCGAC ATCGATTATG ACCATCCTGA TGTCGCAGCA GAAATTAAGA GATGGGGCAC 900

TTGGTATGCC AATGAACTGC AATTGGACGG TTTCCGTCTT GATGCTGTCA AACACATTAA 960

ATTTTCTTTT TTGCGGGATT GGGTTAATCA TGTCAGGGAA AAAACGGGGA AGGAAATGTT 1020

TACGGTAGCT GAATATTGGC AGAATGACTT GGGCGCGCTG GAAAACTATT TGAACAAAAC 1080

AAATTTTAAT CATTCAGTGT TTGACGTGCC GCTTCATTAT CAGTTCCATG CTGCATCGAC 1140

ACAGGGAGGC GGCTATGATA TGAGGAAATT GCTGAACGGT ACGGTCGTTT CCAAGCATCC 1200

GTTGAAATCG GTTACATTTG TCGATAACCA TGATACACAG CCGGGGCAAT CGCTTGAGTC 1260

GACTGTCCAA ACATGGTTTA AGCCGCTTGC TTACGCTTTT ATTCTCACAA GGGAATCTGG 1320

ATACCCTCAG GTTTTCTACG GGGATATGTA CGGGACGAAA GGAGACTCCC AGCGCGAAAT 1380

TCCTGCCTTG AAACACAAAA TTGAACCGAT CTTAAAAGCG AGAAAACAGT ATGCGTACGG 1440

AGCACAGCAT GATTATTTCG ACCACCATGA CATTGTCGGC TGGACAAGGG AAGGCGACAG 1500

CTCGGTTGCA AATTCAGGTT TGGCGGCATT AATAACAGAC GGACCCGGTG GGGCAAAGCG 1560

AATGTATGTC GGCCGGCAAA ACGCCGGTGA GACATGGCAT GACATTACCG GAAACCGTTC 1620

GGAGCCGGTT GTCATCAATT CGGAAGGCTG GGGAGAGTTT CACGTAAACG GCGGGTCGGT 1680

TTCAATTTAT GTTCAAAGAT AGAAGAGCAG AGAGGACGGA TTTCCTGAAG GAAATCCGTT 1740

TTTTTATTTT GCCCGTCTTA TAAATTTCTT TGATTACATT TTATAATTAA TTTTAACAAA 1800

GTGTCATCAG CCCTCAGGAA GGACTTGCTG ACAGTTTGAA TCGCATAGGT AAGGCGGGGA 1860

TGAAATGGCA ACGTTATCTG ATGTAGCAAA GAAAGCAAAT GTGTCGAAAA TGACGGTATC 1920

GCGGGTGATC AATCATCCTG AGACTGTGAC GGATGAATTG AAAAAGCT 1968

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 511 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1               5                   10                  15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
            20                  25                  30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
        35                  40                  45

His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His Gly
    50                  55                  60

Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln Ala
65                  70                  75                  80

Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe His
                85                  90                  95

Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu Gln
            100                 105                 110

Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly Asp
        115                 120                 125

Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val Thr
    130                 135                 140

Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly Glu
145                 150                 155                 160

His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly Ser
                165                 170                 175

Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr Asp
            180                 185                 190

Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly Lys
        195                 200                 205
```

```
Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr Leu
    210                 215                 220

Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu Ile
225                 230                 235                 240

Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly Phe
                245                 250                 255

Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp Trp
            260                 265                 270

Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val Ala
        275                 280                 285

Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys
    290                 295                 300

Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln Phe
305                 310                 315                 320

His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu Leu
                325                 330                 335

Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe Val
            340                 345                 350

Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val Gln
        355                 360                 365

Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser
    370                 375                 380

Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp
385                 390                 395                 400

Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile Leu
                405                 410                 415

Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp
            420                 425                 430

His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val Ala
        435                 440                 445

Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala Lys
    450                 455                 460

Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp Ile
465                 470                 475                 480

Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp Gly
                485                 490                 495

Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
            500                 505                 510
```

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 483 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

```
Ala Asn Leu Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro
1               5                   10                  15

Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu
            20                  25                  30

Ala Glu His Gly Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly
        35                  40                  45

Thr Ser Gln Ala Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu
    50                  55                  60

Gly Glu Phe His Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys
65                  70                  75                  80

Gly Glu Leu Gln Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn
                85                  90                  95

Val Tyr Gly Asp Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr
            100                 105                 110

Glu Asp Val Thr Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val
        115                 120                 125

Ile Ser Gly Glu His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro
    130                 135                 140

Gly Arg Gly Ser Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe
145                 150                 155                 160

Asp Gly Thr Asp Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys
                165                 170                 175
Phe Gln Gly Lys Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn
            180                 185                 190

Tyr Asp Tyr Leu Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val
        195                 200                 205

Ala Ala Glu Ile Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln
    210                 215                 220

Leu Asp Gly Phe Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe
225                 230                 235                 240

Leu Arg Asp Trp Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met
```

```
                245                 250                 255

Phe Thr Val Ala Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn
            260                 265                 270

Tyr Leu Asn Lys Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu
        275                 280                 285

His Tyr Gln Phe His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met
    290                 295                 300

Arg Lys Leu Leu Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser
305                 310                 315                 320

Val Thr Phe Val Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu
                325                 330                 335

Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu
            340                 345                 350

Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly
        355                 360                 365

Thr Lys Gly Asp Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile
    370                 375                 380

Glu Pro Ile Leu Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His
385                 390                 395                 400

Asp Tyr Phe Asp His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp
                405                 410                 415

Ser Ser Val Ala Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro
            420                 425                 430

Gly Gly Ala Lys Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr
        435                 440                 445

Trp His Asp Ile Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser
    450                 455                 460

Glu Gly Trp Gly Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr
465                 470                 475                 480

Val Gln Arg
```

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 511 amino acids

(B) TYPE: amino acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

```
Met Lys Gln Gln Lys Arg Leu Tyr Ala Arg Leu Leu Thr Leu Leu Phe
1               5                   10                  15

Ala Leu Ile Phe Leu Leu Pro His Ser Ala Ala Ala Ala Ala Asn Leu
            20                  25                  30

Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp Tyr Met Pro Asn Asp Gly
        35                  40                  45

His Trp Lys Arg Leu Gln Asn Asp Ser Ala Tyr Leu Ala Glu His Gly
    50                  55                  60

Ile Thr Ala Val Trp Ile Pro Pro Ala Tyr Lys Gly Thr Ser Gln Ala
65                  70                  75                  80

Asp Val Gly Tyr Gly Ala Tyr Asp Leu Tyr Asp Leu Gly Glu Phe His
                85                  90                  95

Gln Lys Gly Thr Val Arg Thr Lys Tyr Gly Thr Lys Gly Glu Leu Gln
            100                 105                 110

Ser Ala Ile Lys Ser Leu His Ser Arg Asp Ile Asn Val Tyr Gly Asp
        115                 120                 125

Val Val Ile Asn His Lys Gly Gly Ala Asp Ala Thr Glu Asp Val Thr
    130                 135                 140

Ala Val Glu Val Asp Pro Ala Asp Arg Asn Arg Val Ile Ser Gly Glu
145                 150                 155                 160

His Leu Ile Lys Ala Trp Thr His Phe His Phe Pro Gly Arg Gly Ser
            165                 170                 175

Thr Tyr Ser Asp Phe Lys Trp His Trp Tyr His Phe Asp Gly Thr Asp
        180                 185                 190

Trp Asp Glu Ser Arg Lys Leu Asn Arg Ile Tyr Lys Phe Gln Gly Lys
    195                 200                 205

Ala Trp Asp Trp Glu Val Ser Asn Glu Asn Gly Asn Tyr Asp Tyr Leu
210                 215                 220                 225

Met Tyr Ala Asp Ile Asp Tyr Asp His Pro Asp Val Ala Ala Glu Ile
                230                 235                 240

Lys Arg Trp Gly Thr Trp Tyr Ala Asn Glu Leu Gln Leu Asp Gly Phe
            245                 250                 255

Arg Leu Asp Ala Val Lys His Ile Lys Phe Ser Phe Leu Arg Asp Trp
        260                 265                 270
Val Asn His Val Arg Glu Lys Thr Gly Lys Glu Met Phe Thr Val Ala
    275                 280                 285

Glu Tyr Trp Gln Asn Asp Leu Gly Ala Leu Glu Asn Tyr Leu Asn Lys
290                 295                 300                 305

Thr Asn Phe Asn His Ser Val Phe Asp Val Pro Leu His Tyr Gln Phe
```

310 315 320

His Ala Ala Ser Thr Gln Gly Gly Gly Tyr Asp Met Arg Lys Leu Leu
325 330 335

Asn Gly Thr Val Val Ser Lys His Pro Leu Lys Ser Val Thr Phe Val
340 345 350

Asp Asn His Asp Thr Gln Pro Gly Gln Ser Leu Glu Ser Thr Val Gln
355 360 365

Thr Trp Phe Lys Pro Leu Ala Tyr Ala Phe Ile Leu Thr Arg Glu Ser
370 375 380 385

Gly Tyr Pro Gln Val Phe Tyr Gly Asp Met Tyr Gly Thr Lys Gly Asp
390 395 400

Ser Gln Arg Glu Ile Pro Ala Leu Lys His Lys Ile Glu Pro Ile Leu
405 410 415

Lys Ala Arg Lys Gln Tyr Ala Tyr Gly Ala Gln His Asp Tyr Phe Asp
420 425 430

His His Asp Ile Val Gly Trp Thr Arg Glu Gly Asp Ser Ser Val Ala
435 440 445

Asn Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly Pro Gly Gly Ala Lys
450 455 460 465

Arg Met Tyr Val Gly Arg Gln Asn Ala Gly Glu Thr Trp His Asp Ile
470 475 480

Thr Gly Asn Arg Ser Glu Pro Val Val Ile Asn Ser Glu Gly Trp Gly
485 490 495

Glu Phe His Val Asn Gly Gly Ser Val Ser Ile Tyr Val Gln Arg
500 505 510

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 520 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

Met Arg Gly Arg Gly Asn Met Ile Gln Lys Arg Lys Arg Thr Val Ser
1 5 10 15

Phe Arg Leu Val Leu Met Cys Thr Leu Leu Phe Val Ser Leu Pro Ile
20 25 30

Thr Lys Thr Ser Ala Val Asn Gly Thr Leu Met Gln Tyr Phe Glu Trp

```
        35                  40                  45

Tyr Thr Pro Asn Asp Gly Gln His Trp Lys Arg Leu Gln Asn Asp Ala
    50                  55                  60

Glu His Leu Ser Asp Ile Gly Ile Thr Ala Val Trp Ile Pro Pro Ala
65                  70                  75                  80

Tyr Lys Gly Leu Ser Gln Ser Asp Asn Gly Tyr Gly Pro Tyr Asp Leu
                85                  90                  95

Tyr Asp Leu Gly Glu Phe Gln Gln Lys Gly Thr Val Arg Thr Lys Tyr
            100                 105                 110

Gly Thr Lys Ser Glu Leu Gln Asp Ala Ile Gly Ser Leu His Ser Arg
        115                 120                 125

Asn Val Gln Val Tyr Gly Asp Val Val Leu Asn His Lys Ala Gly Ala
    130                 135                 140

Asp Ala Thr Glu Asp Val Thr Ala Val Glu Val Asn Pro Ala Asn Arg
145                 150                 155                 160

Asn Gln Glu Thr Ser Glu Glu Tyr Gln Ile Lys Ala Trp Thr Asp Phe
                165                 170                 175

Arg Phe Pro Gly Arg Gly Asn Thr Tyr Ser Asp Phe Lys Trp His Trp
            180                 185                 190

Tyr His Phe Asp Gly Ala Asp Trp Asp Glu Ser Arg Lys Ile Ser Arg
        195                 200                 205

Ile Phe Lys Phe Arg Gly Glu Gly Lys Ala Trp Asp Trp Glu Val Ser
    210                 215                 220

Ser Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Val Asp Tyr
225                 230                 235                 240

Asp His Pro Asp Val Val Ala Glu Thr Lys Lys Trp Gly Ile Trp Tyr
                245                 250                 255

Ala Asn Glu Leu Ser Leu Asp Gly Phe Arg Ile Asp Ala Ala Lys His
            260                 265                 270

Ile Lys Phe Ser Phe Leu Arg Asp Trp Val Gln Ala Val Arg Gln Ala
        275                 280                 285

Thr Gly Lys Glu Met Phe Thr Val Ala Glu Tyr Trp Gln Asn Asn Ala
    290                 295                 300

Gly Lys Leu Glu Asn Tyr Leu Asn Lys Thr Ser Phe Asn Gln Ser Val
305                 310                 315                 320

Phe Asp Val Pro Leu His Phe Asn Leu Gln Ala Ala Ser Ser Gln Gly
                325                 330                 335

Gly Gly Tyr Asp Met Arg Arg Leu Leu Asp Gly Thr Val Val Ser Arg
```

340 345 350

His Pro Glu Lys Ala Val Thr Phe Val Glu Asn His Asp Thr Gln Pro
355 360 365

Gly Gln Ser Leu Glu Ser Thr Val Gln Thr Trp Phe Lys Pro Leu Ala
370 375 380

Tyr Ala Phe Ile Leu Thr Arg Glu Ser Gly Tyr Pro Gln Val Phe Tyr
385 390 395 400

Gly Asp Met Tyr Gly Thr Lys Gly Thr Ser Pro Lys Glu Ile Pro Ser
405 410 415

Leu Lys Asp Asn Ile Glu Pro Ile Leu Lys Ala Arg Lys Glu Tyr Ala
420 425 430

Tyr Gly Pro Gln His Asp Tyr Ile Asp His Pro Asp Val Ile Gly Trp
435 440 445

Thr Arg Glu Gly Asp Ser Ser Ala Ala Lys Ser Gly Leu Ala Ala Leu
450 455 460

Ile Thr Asp Gly Pro Gly Gly Ser Lys Arg Met Tyr Ala Gly Leu Lys
465 470 475 480

Asn Ala Gly Glu Thr Trp Tyr Asp Ile Thr Gly Asn Arg Ser Asp Thr
485 490 495

Val Lys Ile Gly Ser Asp Gly Trp Gly Glu Phe His Val Asn Asp Gly
500 505 510

Ser Val Ser Ile Tyr Val Gln Lys
515 520

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 548 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: unknown

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

Val Leu Thr Phe His Arg Ile Ile Arg Lys Gly Trp Met Phe Leu Leu
1 5 10 15

Ala Phe Leu Leu Thr Ala Ser Leu Phe Cys Pro Thr Gly Arg His Ala
20 25 30

Lys Ala Ala Ala Pro Phe Asn Gly Thr Met Met Gln Tyr Phe Glu Trp
35 40 45

Tyr Leu Pro Asp Asp Gly Thr Leu Trp Thr Lys Val Ala Asn Glu Ala

50 55 60

Asn Asn Leu Ser Ser Leu Gly Ile Thr Ala Leu Ser Leu Pro Pro Ala
65 70 75 80

Tyr Lys Gly Thr Ser Arg Ser Asp Val Gly Tyr Gly Val Tyr Asp Leu
85 90 95

Tyr Asp Leu Gly Glu Phe Asn Gln Lys Gly Thr Val Arg Thr Lys Tyr
100 105 110

Gly Thr Lys Ala Gln Tyr Leu Gln Ala Ile Gln Ala Ala His Ala Ala
115 120 125

Gly Met Gln Val Tyr Ala Asp Val Val Phe Asp His Lys Gly Gly Ala
130 135 140

Asp Gly Thr Glu Trp Val Asp Ala Val Glu Val Asn Pro Ser Asp Arg
145 150 155 160

Asn Gln Glu Ile Ser Gly Thr Tyr Gln Ile Gln Ala Trp Thr Lys Phe
165 170 175

Asp Phe Pro Gly Arg Gly Asn Thr Tyr Ser Ser Phe Lys Trp Arg Trp
180 185 190

Tyr His Phe Asp Gly Val Asp Trp Asp Glu Ser Arg Lys Leu Ser Arg
195 200 205

Ile Tyr Lys Phe Arg Gly Ile Gly Lys Ala Trp Asp Trp Glu Val Asp
210 215 220

Thr Glu Asn Gly Asn Tyr Asp Tyr Leu Met Tyr Ala Asp Leu Asp Met
225 230 235 240

Asp His Pro Glu Val Val Thr Glu Leu Lys Asn Trp Gly Lys Trp Tyr
245 250 255

Val Asn Thr Thr Asn Ile Asp Gly Phe Arg Leu Asp Gly Leu Lys His
260 265 270

Ile Lys Phe Ser Phe Phe Pro Asp Trp Leu Ser Tyr Val Arg Ser Gln
275 280 285

Thr Gly Lys Pro Leu Phe Thr Val Gly Glu Tyr Trp Ser Tyr Asp Ile
290 295 300

Asn Lys Leu His Asn Tyr Ile Thr Lys Thr Asn Gly Thr Met Ser Leu
305 310 315 320

Phe Asp Ala Pro Leu His Asn Lys Phe Tyr Thr Ala Ser Lys Ser Gly
325 330 335

Gly Ala Phe Asp Met Arg Thr Leu Met Thr Asn Thr Leu Met Lys Asp
340 345 350

Gln Pro Thr Leu Ala Val Thr Phe Val Asp Asn His Asp Thr Asn Pro
355 360 365

```
Ala Lys Arg Cys Ser His Gly Arg Pro Trp Phe Lys Pro Leu Ala Tyr
    370                 375                 380

Ala Phe Ile Leu Thr Arg Gln Glu Gly Tyr Pro Cys Val Phe Tyr Gly
385                 390                 395                 400

Asp Tyr Tyr Gly Ile Pro Gln Tyr Asn Ile Pro Ser Leu Lys Ser Lys
                405                 410                 415

Ile Asp Pro Leu Leu Ile Ala Arg Arg Asp Tyr Ala Tyr Gly Thr Gln
            420                 425                 430

His Asp Tyr Leu Asp His Ser Asp Ile Ile Gly Trp Thr Arg Glu Gly
        435                 440                 445

Val Thr Glu Lys Pro Gly Ser Gly Leu Ala Ala Leu Ile Thr Asp Gly
    450                 455                 460

Ala Gly Arg Ser Lys Trp Met Tyr Val Gly Lys Gln His Ala Gly Lys
465                 470                 475                 480

Val Phe Tyr Asp Leu Thr Gly Asn Arg Ser Asp Thr Val Thr Ile Asn
                485                 490                 495

Ser Asp Gly Trp Gly Glu Phe Lys Val Asn Gly Gly Ser Val Ser Val
            500                 505                 510

Trp Val Pro Arg Lys Thr Thr Val Ser Thr Ile Ala Arg Pro Ile Thr
        515                 520                 525

Thr Arg Pro Trp Thr Gly Glu Phe Val Arg Trp His Glu Pro Arg Leu
    530                 535                 540

Val Ala Trp Pro
545
```

(2) INFORMATION FOR SEQ ID NO: 7

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 56 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 7:

```
GATCAAAACA TAAAAAACCG GCCTTGGCCC CGCCGGTTTT TTATTATTTT TGAGCT              56
```

(2) INFORMATION FOR SEQ ID NO: 8:

(i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 48 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 8:

CAAAAATAAT AAAAAACCGG CGGGGCCAAG GCCGGTTTTT TATGTTTT 48

(2) INFORMATION FOR SEQ ID NO: 9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 38 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 9:

CCCATTAAGA TTGGCCGCCT GGGCCGACAT GTTGCTGG 38

(2) INFORMATION FOR SEQ ID NO: 10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

CCAGCCGACA ATGTCATGGT CGTCGAAATA ATC 33

(2) INFORMATION FOR SEQ ID NO: 11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

CCTGATGTCG CAACAGAAAT TAAGAGATGG 30

(2) INFORMATION FOR SEQ ID NO: 12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs

(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

GTCGCCTTCC CTTGCCCAGC CGACAATGTC 30

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 98/16906

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/56 C12N9/28 C12Q1/40 C11D3/386

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C12Q C11D

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 23874 A (NOVO NORDISK A/S (DK); SVENDSEN A.; BISGAARD-FRANTZEN H.; BORCHERT T.V) 8 August 1996 cited in the application	1,4-7, 9-15
Y	see page 30, line 8-34 see page 33, line 33 - page 34, line 2 see page 47, line 12-16 ---	7,8
Y	WO 96 30481 A (GENENCOR INTERNATIONAL INC (US); BARNETT; BOYER; MITCHINSON; POWER) 3 October 1996 see page 5, paragraph 3 - page 7, line 6 see page 71 - page 72; claims ---	7,8

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
25 November 1998	03/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Macchia, G

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

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